Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1

ERIC YUNG[1], MASHA SORIN[1], ACHINTYA PAL[1], ERROL CRAIG[1], ALEXEI MOROZOV[1], OLIVIER DELATTRE[4], JOHN KAPPES[3], DAVID OTT[2] & GANJAM V. KALPANA[1]

[1]*Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York, USA*
[2]*AIDS Vaccine Program, SAIC-Frederick, National Cancer Institute-Frederick, Frederick, Maryland, USA*
[3]*Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA*
[4]*Laboratoire de Pathologie Moleculaire des Cancers, Institut Curie, Paris, France*
Correspondence should be addressed to G.V.K.; email: kalpana@aecom.yu.edu

Integase interactor 1 (INI1), also known as hSNF5, is a protein that interacts with HIV-1 integrase. We report here that a cytoplasmically localized fragment of INI1 (S6; aa183–294) containing the minimal integrase-interaction domain potently inhibits HIV-1 particle production and replication. Mutations in S6 or integrase that disrupt integrase–INI1 interaction abrogated the inhibitory effect. An integrase-deficient HIV-1 transcomplemented with integrase fused to Vpr was not affected by S6. INI1 was specifically incorporated into virions and was required for efficient HIV-1 particle production. These results indicate that INI1 is required for late events in the viral life cycle, and that ectopic expression of S6 inhibits HIV-1 replication in a transdominant manner via its specific interaction with integrase within the context of Gag–Pol, providing a novel strategy to control HIV-1 replication.

Although multi-drug therapy is an effective treatment for HIV-1 infection, viral relapses occur in at least half of the patients due to incomplete adherence to drug regimen leading to the emergence of drug resistant variants. This problem necessitates development of new therapeutic agents to target HIV-1. HIV-1 proteins such as integrase and the cellular proteins implicated in viral replication are attractive new targets[1–3]. Integrase catalyses integration of HIV-1 DNA into the host genome, an essential step in HIV-1 replication[4–6]. Integrase consists of an N-terminal zinc-finger domain, a catalytic core domain with a conserved D,D(35)E motif and a C-terminal domain[7]. *In vivo* studies using molecular clones of HIV-1 have shown that integrase mutations have pleiotropic effects, blocking other steps of viral replication in addition to integration[8–12]. Understanding the mechanisms of these pleiotropic effects might lead to the development of antiviral drugs that affect multiple steps of the HIV-1 lifecycle by targeting integrase.

Integrase interactor 1 (INI1), also known as hSNF5, isolated using a yeast two-hybrid screen[13,14], is the only known host protein that directly interacts with HIV-1 integrase. It is a tumor suppressor and a core component of the SWI/SNF complex involved in chromatin remodeling[15–18]. It has three highly conserved domains, two of which are imperfect repeats (Rpt1 and Rpt2). HIV-1 integrase specifically binds to Rpt1 but not Rpt2 of INI1 despite their similarity[14]. Given that integrase has pleiotropic effects, we sought to identify fragments of INI1 that bind to integrase and interfere with any of the steps in HIV-1 replication. We report here that expression of a minimal integrase-binding fragment of INI1 (S6; comprising aa183–294) potently inhibits HIV-1 assembly, particle production and replication in a transdominant manner. Genetic and biochemical analyses indicate that this effect results from direct interaction of S6 with integrase within the context of Gag–Pol *in vivo*. Moreover, INI1 is incorporated into the virions and is necessary for efficient particle production. These observations indicate that INI1 is important for HIV-1 replication and provide novel strategies for developing antivirals.

S6 Inhibits HIV-1 particle production and replication

We generated a panel of INI1 truncations containing the minimal integrase-interaction domain (Rpt1) as hemagglutinin (HA) fusions (Fig. 1*a*). We confirmed their expression upon transient transfection into 293T cells, and first tested their effect on post-integration events of HIV-1 replication leading to assembly and budding. We co-expressed HA-tagged INI1 fragments along with a three-plasmid–based HIV-1 vector system[19] in 293T cells and monitored the particle production by p24 ELISA of the culture supernatants. Whereas full-length INI1 had no significant effect, different truncation mutants affected particle production to varying degrees (Table 1). We focused on S6 as it reduced viral production to undetectable levels (Table 1). Moreover, HA-S6 but not full-length HA-INI1 potently inhibited particle production of HIV-1_{R3B}, a replication-competent molecular clone, in transfected 293T cells (Table 2).

To exclude the possibility that the decrease in viral particle production is due to the effect of HA-S6 on the intracellular viral protein synthesis, we examined the levels of both the intracellular and virion-associated p24. Multiple experiments confirmed that 293T cells co-transfected with S6 contained similar intracellular p24 levels compared with cells co-transfected with INI1 (Fig. 1*b*). Nevertheless, p24 levels in the culture supernatants from cells co-transfected with S6 were substantially reduced and consistently yielded 10,000–100,000 times less p24 antigen than the controls (Fig. 1*b*). Thus inhibition of particle production by HA-S6 was not due to a decrease in the intracellular levels of viral proteins.

Table 1 Inhibition of HIV-1 virion production by INI1 truncations

Plasmids	Protein expression[a]	P24 of culture supernatants (ng/ml)	% inhibition[b]
Mock	N/A	0.00	N/A
Viral vectors[c]	N/A	113.28	0%
Viral vectors+pCGN-INI1	++	115.05	0%
Viral vectors+pCGN-27B (HA-ΔINI1 aa1–245)	++	95.57	16%
Viral vectors+pCGN-20.2 (HA-ΔINI1 aa141–385)	++	35.37	69%
Viral vectors+pCGN-S6 (HA-ΔINI1 aa183–294)	+++	0.00	100%

[a], Expression of INI1 and its truncations were determined by immunoblot analysis using α-HA antibodies. [b], Amount of p24 produced with viral vectors alone was considered to be 100%. The inhibition is expressed as 0% for this sample. The percent inhibition in p24 levels in other samples was calculated relative to that of viral vectors alone. [c], Viral vectors are pCMVΔR8.2, pMDG and pHR′-CMV-LacZ. + indicates relative levels of expression of proteins.

To determine if inhibition of late viral events by HA-S6 is sufficient to abrogate the spread of the replication-competent HIV-1 through natural target cells, we generated a pool of Jurkat T-cell clones stably expressing HA-S6, and we infected them with either 0.3 or 1.67 multiplicity of infection (m.o.i.) of HIV-1_{R3B}. We monitored the viral spread by assaying reverse transcriptase (RT) activity in culture supernatants (Fig. 1*c*). Whereas the control Jurkat cells supported infection by HIV-1_{R3B} that reached a peak production at day 10, the pool of Jurkat cell clones expressing HA-S6 showed significantly decreased levels of viral replication even after day 14 of culture. Residual replication of HIV-1_{R3B} seen in pools of cells expressing HA-S6 is probably due to the presence of cells expressing little or no HA-S6. Therefore the dominant-negative mutant is effective in protecting T-cells from infection by HIV-1.

Mutations in S6 abrogate the inhibitory effect

To determine if inhibition of particle production by HA-S6 is due to its specific interaction with integrase, we isolated integrase-interaction–defective mutants of S6. We generated a library of S6 mutants using PCR-based random mutagenesis[20]. We then used a reverse yeast two-hybrid system to screen this library for mutants of S6 that do not interact with a LexA DNA-binding domain fusion of integrase (pSH2–integrase)[14]. Of the several integrase-interaction–defective mutants, four had single amino-acid substitutions (E3, E4, E7 or E10; Fig. 2*a*). All four mutations were located within the C-terminal half of the Rpt1 portion of S6, which is encoded by a single exon, indicating that these mutations are present within one functional domain (Fig. 2*a*).

To test if the integrase-interaction–defective variant of S6 would result in a reduced inhibition of particle production, we co-transfected three-plasmid-based HIV-1 vectors into 293T cells with full-length INI1, HA-S6 or HA-S6 mutants and measured both intra-cellular and particle-associated p24. There was no significant difference in intracellular p24 levels between the cells co-transfected with HA-S6 or integrase-interaction–defective HA-S6 mutants. As before, HA-S6 severely reduced the levels of extracellular p24. However, each of the four mutants resulted in the reversal of inhibition of extracellular p24 production compared with that of HA-S6 (Fig. 2*b*). The strongest reversal of inhibition (a 2-fold inhibition as opposed to 80,000-fold with HA-S6) was that of the HA-E3 mutant carrying a glycine substitution at the invariant D224 residue (Fig. 2*b*). We determined the number of infectious units per milliliter in culture supernatants produced in 293T cells expressing HA-INI1, HA-S6 or HA-E3 by using cytomegalovirus (CMV)-LacZ markers[30] in the transducing vector. Whereas HA-S6 expression led to nearly complete abrogation of viral infectivity, HA-E3 expression led to a mere two-fold reduction in infectivity compared with the control where empty vector was expressed (Fig. 2*c*). Thus loss of inhibition is correlated with the increased production of infectious virus particles.

Immunoblot analysis of lysates from cells co-transfected with HA-S6 or its mutants, along with HIV-1 based vectors, showed that S6 and all four point mutants were expressed at equivalent levels (Fig. 2*d*, bottom). In addition, immunoblot analysis of the same lysates using an antibody specific for integrase to determine the levels of Gag–Pol indicated no significant increase of this polyprotein in cells transfected with E3, E4, E7 or E10 as compared with S6 (Fig. 2*d*, top). Therefore reversal of inhibition is probably not due to an increase in Gag–Pol protein in the presence of S6 mutants.

To confirm that the S6 mutants do not interact with integrase expressed as part of Pol *in vivo*, we co-transfected 293T cells with plasmids expressing HA-INI1, HA-S6 or HA-E3 along with Vpr-RT-integrase (ref. 21). In addition, to assess any potential effect of other viral proteins such as Gag or Env on this interaction, we co-transfected SG3-integrase, a molecular clone of HIV-1 with a premature stop codon at the end of RT. We performed immunoprecipitation using an antibody against HA. The precipitated proteins were probed with antibodies against integrase and HA. Whereas both HA-INI1 and HA-S6 were able to co-immunoprecipitate Vpr-RT-integrase, the S6 mutant HA-E3 was not, though it was present at similar or higher levels in the lysate (Fig. 2*e*). This result indicates for the first time that INI1 and S6 can interact with integrase *in vivo* and confirms that E3 is a true interaction-defective mutant of S6.

Integrase mutation abrogates S6-mediated inhibition

The loss of inhibition by integrase-interaction-defective mutants of S6 indicated that the integrase-S6 interaction is necessary for the inhibitory effect. Similarly, viruses carrying INI1-interaction–defective mutations in integrase might not be inhibited by S6. To test this, we isolated an INI1-interaction–defective mutant of integrase using reverse yeast two-hybrid screen (A.P. and G.V.K., unpublished observations). One of the clones recovered had a point mutation that resulted in substitution of the histidine at position 12 to tyrosine (H12Y). We performed an *in vitro* glutathione-S-transferase (GST) pull-down assay to confirm that

Table 2 Inhibition of R3B virion production by S6

Plasmids	P24 of culture supernatants (ng/ml)	% inhibition*
Mock	0.00	N/A
R3B	> 264.09	0%
R3B + pCGN-INI1	> 264.09	0%
R3B + pCGN-S6 (HA-ΔINI1 aa183–294)	0.69	> 99.74%

*, Amount of p24 produced with R3B alone was considered to be 100%. The inhibition is expressed as 0% for this sample. The % inhibition in p24 levels in other samples was calculated relative to that of R3B alone.

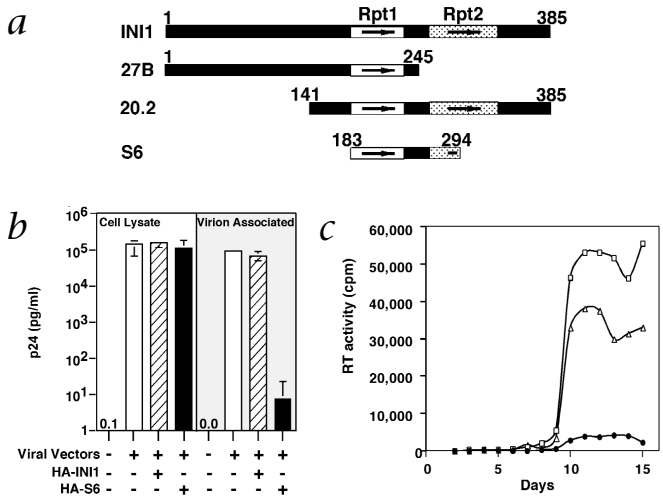

Fig. 1 S6 inhibits HIV-1 particle production and replication. ***a***, Schematic diagram of INI1 truncations used. Names of clones are indicated. Number above the bars represents aa residues. ***b***, Logarithmic graph of intracellular and virion-associated p24 antigen (pg/ml) in the presence and absence of HA-INI1 or HA-S6 (average of three independent experiments).□, viral vectors; ▨, viral vectors+HA-INI1; ■, viral vectors +HA-S6. ***c***, Effect of S6 on the replication HIV-1_{R3B} in Jurkat T-cells. The graph represents RT activity of the culture supernatants infected with HIV-1_{R3B}. △, Jurkat control; □, pools of Jurkat T-cells stably expressing HA-INI; ●, pools of Jurkat T-cells stably expressing HA-S6. Each data point represents RT activity of about 8 µl of culture supernatant.

the H12Y integrase mutant is defective for interaction with INI1 (Fig. 3*a*). Consistent with the yeast two-hybrid analysis, we found that the H12Y mutant had greatly reduced binding to INI1 *in vitro* compared with wild-type integrase, despite similar levels of input proteins (Fig. 3*a*, compare lanes 3 and 6). These results confirm that the mutation in the zinc-finger domain disrupts integrase–INI1 interactions.

We incorporated the H12Y mutation into the pCMVΔR8.2 plasmid that encodes Gag–Pol polyprotein of the three-plasmid–based HIV-1 vector. The effect of HA-INI1, HA-S6 or HA-E3 on the H12Y mutant virus production in 293T cells was tested as before by co-transfections. The intra-cellular p24 levels of H12Y virus were similar to that of the wild-type virus (compare Figs. 1*b* and 3*b*). There was a uniform 10-fold decrease in the extracellular particle-associated p24 levels of H12Y virus compared with that of the corresponding intracellular p24 levels (Fig. 3*b*). These results indicate that the H12Y mutation by itself decreases the particle production 10-fold. Repeated experiments indicated that co-transfection of HA-S6 resulted in an approximately 10-fold decrease in the level of H12Y virion-associated p24 production as opposed to a 10,000–100,000-fold decrease in p24 production occurring with wild-type (Fig. 3*b*). This 10-fold decrease in virion-associated p24 of H12Y virus might be due to a corresponding reduction in the intracellular p24 levels in the presence of HA-S6 (Fig. 3*b*). Together, these results indicate that introduction of an INI1-interaction defective mutation into integrase significantly abrogates the inhibitory effect of S6.

S6-mediated inhibition requires integrase within Gag–Pol

Certain integrase substitution mutations affect HIV-1 particle production probably due to their effect on the processing of Gag–Pol (refs. 22–27). These effects could be overcome if integrase is removed from Gag–Pol and integrase mutants are pro-

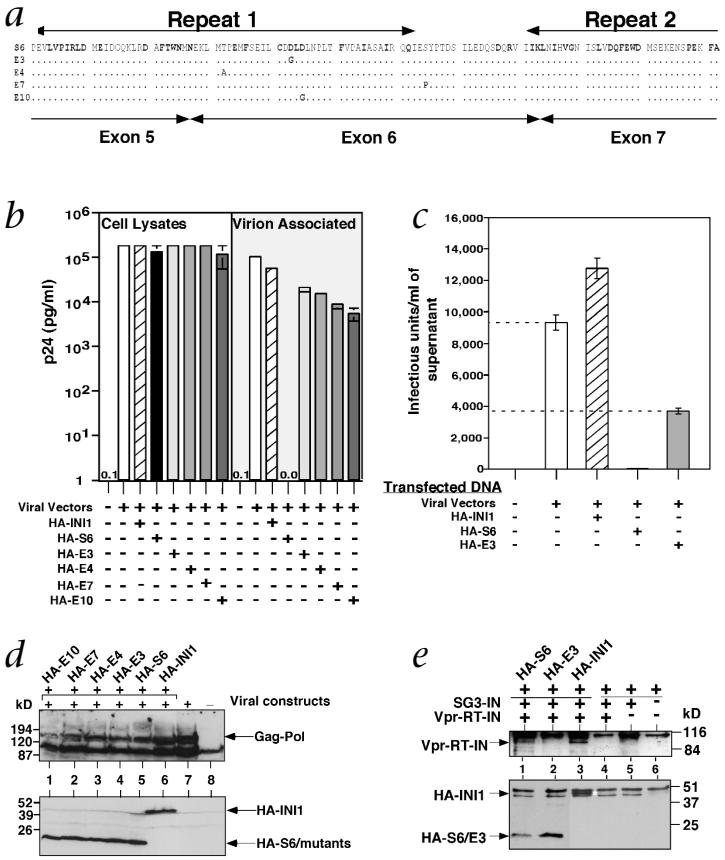

Fig. 2 Interaction-defective mutants of S6 abrogate the inhibitory effect on viral particle production. ***a***, Location of substitution mutations found in the integrase-interaction–defective mutants of S6. The highly conserved residues of INI1 are indicated in bold. Repeat 1 and Repeat 2 represent the two highly conserved domains of INI1. The regions of S6 encoded by specific exons are indicated at the bottom. Substitutions in S6 mutants, E3–E10 are indicated. ***b***, Integrase-interaction–defective mutants of S6 abrogate the inhibition of particle production by S6. Logarithmic graph of intracellular and virion-associated p24 antigen (pg/ml) in the presence and absence of HA-INI1, HA-S6 and HA-E3–E10 (average of 3 independent experiments). □, viral vectors; ▨, viral vectors+HA-INI1; ■, viral vectors+HA-S6; ▩, viral vectors+HA-E3; ▩, viral vectors+HA-E4; ▩, viral vectors+HA-E7; ■, viral vectors +HA-E10. ***c***, Infectivity of virus produced in the presence and absence of HA-INI1, HA-S6 or HA-E3. Infectious units refers to the number of $LacZ^+$ cells obtained after staining with X-gal, when infected with culture supernatants carrying pseudotyped HIV-1-LacZ virus. ***d***, Immunoblot analysis of relative amounts of HA-INI1, HA-S6 or HA-E3–E10 and Gag-Pol in the producer cells. Immunoblot using antibodies against integrase (top) or HA (bottom). ***e***, Co-immunoprecipitation analysis of the association of HA-INI1, HA-S6 and HA-E3 with Vpr-RT-integrase *in vivo*. Plasmids transfected into 293T cells are indicated. The immunoprecipitations were performed using antibody against HA and immunoblotted with a monoclonal antibody against integrase (top) to determine the co-immunoprecipitation of Vpr-RT-integrase or against HA (bottom) to determine the levels of HA-INI1, HA-S6 or HA-E3 in the immunoprecipitates. White *, band that corresponds to HA-INI1.

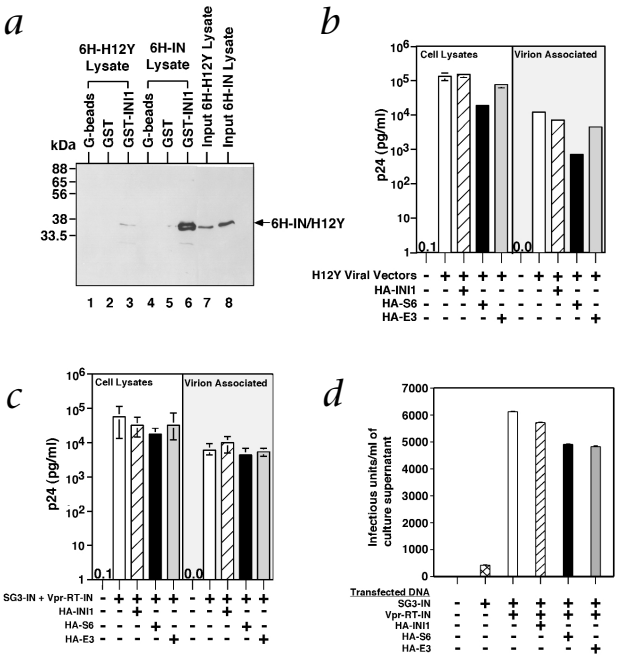

Fig. 3 INI1-interaction–defective mutation in integrase abrogates the inhibitory effect of S6. ***a***, *In vitro* binding studies of GST-INI1 with 6H-integrase and 6H-H12Y. Panel shows immunoblot analysis of the proteins bound to GST proteins, using antibodies against 6H. G-beads, glutathione agarose beads. Lanes 1–3, binding of GST-INI1 to 6H-H12Y; lanes 4–6, binding of GST-INI1 protein to 6H-integrase. ***b***, H12Y mutation abrogates the inhibitory effect of S6. Logarithmic graph of intracellular and virion-associated p24 antigen (pg/ml) of H12Y virus in the presence and absence of HA-INI1, HA-S6 or HA-E3 (average of 3 independent experiments). Columns are labeled as in Fig. 2*b*. ***c*** and ***d***, S6 does not affect the virus production of an integrase-deficient HIV-1. HIV-1 virus (SG3-integrase) containing Vpr-RT-integrase was produced in the presence and absence of HA-INI1, HA-S6 or HA-E3 (*c*). Logarithmic graph of intracellular and virion associated p24 antigen (pg/ml) of SG3-IN+Vpr-RT-integrase in the presence and absence of HA-INI1, -S6 or -E3 (average of 3 independent experiments). Graph shows numbers of LacZ$^+$ cells obtained after infecting P4 (HeLa/CD4$^+$/LTR-LacZ) cells with culture supernatants containing SG3-IN+Vpr-RT-integrase virus produced in the presence and absence of HA-INI1, HA-S6 and HA-E3 (*d*).

vided *in trans* as Vpr or Vpr-RT fusions[21,28]. We surmised that binding of S6 to integrase might mimic the effects of these integrase mutations. We therefore tested the effect of S6 on particle production of the SG3-integrase virus that carries a stop codon at the end of RT, and *trans*-complemented integrase as a Vpr-RT–integrase fusion. The results indicated that there was no significant difference in the intracellular p24 levels, and with integrase no longer being a part of Gag–Pol, there was no significant difference in the extracellular p24 levels with or without S6 (Fig. 3*c*). Furthermore, no significant differences were noticed in the infectivity of the culture supernatants in the presence and absence of HA-S6 when assayed using P4 (HeLa CD4$^+$/LTR-LacZ) cells[21] (Fig. 3*d*). These results indicate that inhibition by S6 requires integrase to be part of Gag–Pol.

INI1 is nuclear but S6 is predominantly cytoplasmic

The above results indicate that S6 inhibits viral particle production by directly interacting with integrase, and by interfering with some steps of the assembly or budding processes that involve Gag–Pol. However, INI1 is a nuclear protein (ref. 29 and E.C. and G.V.K., manuscript submitted), and these late events occur in the cytoplasm. To reconcile this problem, we hypothesized that S6 might be localized in the cytoplasm and that the dominant-negative effects of S6 may be due to its ectopic expression. To test this, we expressed INI1, S6 and the interaction-defective mutant E3 as green fluorescent protein (GFP)-fusion proteins in 293T cells and observed their sub-cellular localization by confocal microscopy. We observed that whereas INI1 is nuclear, S6 is predominantly cytoplasmic (Fig. 4). Interestingly, the integrase-interaction-defective S6 mutant E3 also exhibited cytoplasmic localization (Fig. 4), indicating that mere cytoplasmic localization is not sufficient for transdominant inhibition and that interaction with integrase is required.

INI1 is required for efficient HIV-1 production

The interference of late events by S6 means that INI1 might be important for these stages of HIV-1 replication. To determine if INI1 is indeed required for late events in HIV-1 particle production, we tested the MON cell line that carries a homozygous deletion of the gene encoding INI1 (ref. 15) for its ability to support HIV-1 particle production. We found that when three-plasmid–based vectors were introduced into MON cells, both p24 production and infectivity were significantly reduced (~10–100-fold decrease compared with 293T cells). To determine if the decrease is due to the lack of INI1, we co-transfected MON cells with increasing concentrations of INI1 along with constant amounts of viral vectors. Our results indicate that although co-expression of INI1 only slightly increased the p24 levels within MON cells, it significantly rescued the defect in viral particle production in a dose-dependent manner (Fig. 5*a*). This increase in p24 production correlated with a corresponding increase in the number of infectious particles in the culture supernatants (Fig. 5*a*), indicating that INI1 is required for the efficient production of infectious virions.

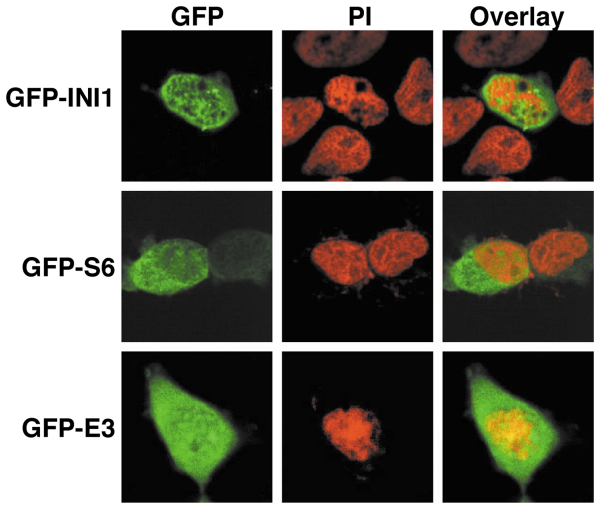

Fig. 4 Sub-cellular localization of INI1 and the truncation mutants S6 and E3. GFP, GFP-INI1, GFP-S6 or GFP-E3 represent the fusion proteins expressed in transfected 293T cells. The left panels of each row depict the fluorescence due to GFP, the middle panels depict the propidium iodide staining of the nuclei and the right panels depict the overlay.

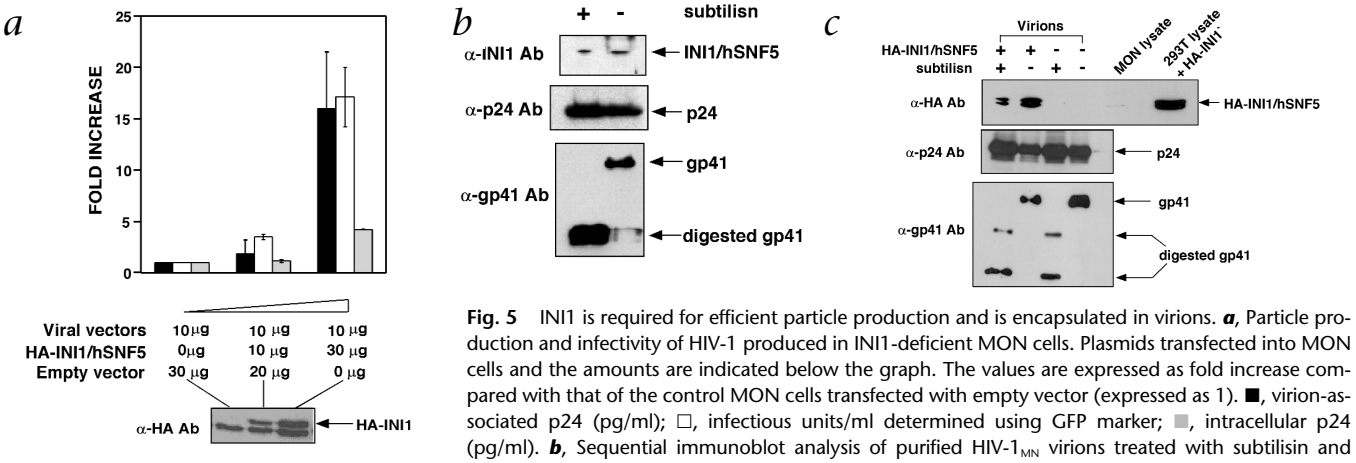

Fig. 5 INI1 is required for efficient particle production and is encapsulated in virions. ***a***, Particle production and infectivity of HIV-1 produced in INI1-deficient MON cells. Plasmids transfected into MON cells and the amounts are indicated below the graph. The values are expressed as fold increase compared with that of the control MON cells transfected with empty vector (expressed as 1). ■, virion-associated p24 (pg/ml); □, infectious units/ml determined using GFP marker; ■, intracellular p24 (pg/ml). ***b***, Sequential immunoblot analysis of purified HIV-1_{MN} virions treated with subtilisin and probed with antibodies against INI1, gp41 or gp24. α, anti-; Ab, antibody. ***c***, Incorporation of tagged HA-INI1 into HIV-1_{R3B}. Immunoblot analysis of subtilisin treated HIV-1_{R3B} virus produced from 293T cells in the presence and absence of HA-INI1.

INI1 is incorporated into HIV-1 virions

Pseudotyped HIV-1 particles, produced from the 293T cells, were able to efficiently infect MON cells, suggesting that these INI1-deficient cells support early events in HIV-1 replication. One possible explanation for this result is that INI1 present in 293T cells is packaged into the virions and is sufficient for inducing early events in INI1-deficient MON cells. To test this hypothesis, we purified and concentrated HIV-1_{MN} virions by sucrose-density gradient sedimentation and subjected them to subtilisin treatment[30]. We subjected the total proteins from this treatment to successive rounds of immunoblot analysis with antibodies against INI1, gp41 (SU) and p24 (Fig. 5*b*). We observed that similar amounts of INI1 are present in both untreated and subtilisin-treated virions. Use of antibody against gp41 revealed that subtilisin treatment was complete in these samples. To further confirm this observation, we examined the ability of tagged INI1 to be incorporated into HIV-1_{R3B} virions. The virus particles, produced from the 293T cells in the presence or absence of transiently transfected HA-INI1, were subjected to the subtilisin treatment and immunoblot analysis as above (Fig. 5*c*). These data indicate that HA-INI1 is specifically incorporated into virions. These results show for the first time that HIV-1 virions encapsidate INI1.

Discussion

We have demonstrated a novel method of inhibiting HIV-1 replication using S6, a 111-aa fragment of INI1. This fragment strongly inhibited HIV-1 particle production in a dominant-negative manner, reducing the p24 amount by more than 10,000-fold. S6 also inhibited the spread of replication-competent HIV-1 in T-cell cultures. We show that this inhibition is mediated by a direct interaction of S6 with integrase within the context of Gag–Pol. First, integrase-interaction–defective mutants of S6 that we isolated did not display drastic inhibition of particle production. Second, viruses carrying an INI1-interaction–defective mutant of integrase (H12Y) were not significantly inhibited by S6. And third, removal of integrase from Gag–Pol resulted in the abrogation of this inhibition. Moreover, we found that unlike the nuclear INI1, S6 predominantly localizes to the cytoplasm, suggesting that its ectopic expression contributes to the inhibitory effect. To our knowledge, this is the first example of a truncation fragment of a cellular protein inhibiting late events of HIV-1 replication via integrase.

It has been reported that although Pol or integrase is not necessary for the assembly and budding of HIV-1, mutations in integrase have severe effects on these processes[8,11,27,31]. The binding of S6 with the integrase portion of Gag–Pol might mimic the effects of such integrase mutations and therefore could similarly block some steps of late events. We propose that when S6 binds to integrase, it might interfere with the proper multimerization of Gag and Gag–Pol by steric hindrance, affect maturation, block an interaction of the cellular assembly machinery with Gag–Pol or mediate the mislocalization of viral proteins into a different sub-cytoplasmic compartment. Removing integrase from Gag–Pol and providing it *in trans* as a Vpr-fusion overcomes these defects, as S6 can no longer physically interact with Gag–Pol deleted of integrase. Interestingly, packaging of Vpr into mature virions might involve mechanisms distinct from that for Gag–Pol and hence Vpr-RT-integrase incorporation is not inhibited by S6. Currently, the molecular mechanisms underlying virus assembly, packaging, egress and the host factors involved in these processes are not fully understood[32]. Future studies using S6 might help elucidate these mechanisms.

Our results indicate that INI1 is important for HIV-1 replication. In addition to the dominant-negative effect of the INI1 mutant, we show for the first time that INI1 is packaged in virions. Moreover, we have found that there is reduced particle production in a cell line homozygously deleted for the gene encoding INI1, and that INI1 expression corrects this defect in a dose-dependent manner. The observed 10-fold decrease in particle production of INI1-interaction–defective mutant H12Y indicates that the integrase-INI1 interaction has a role in these processes. Our attempts to study the effect of S6 on early events resulted in no significant inhibition (data not shown), probably because of the cytoplasmic localization of S6. However, this data does not preclude the possibility that full-length INI1 influences early events in viral replication.

The identification of a small fragment of a cellular protein that acts as a potent dominant-negative inhibitor especially of the late events of the HIV-1 life cycle provides exciting possibilities for therapeutic intervention of AIDS. S6 does not appear to be cytotoxic as cells stably expressing S6 grow normally (data not

shown). Because S6 is a fragment of a host protein, it is unlikely to be immunogenic. In addition, mutations that make integrase defective for interaction with INI1 or S6 might also render the virus replication-defective, thus making it harder to develop resistance to S6. Gene therapy to deliver S6 or its derivatives to hematopoietic stem cells could be a useful strategy to control HIV-1 infection. Moreover, the profound inhibition of late events by S6 makes it an attractive candidate for developing effective low-molecular-weight drugs for controlling re-emergence of HIV-1 from latently infected cells.

Methods

Construction and expression of INI1 truncations in mammalian cells. Generation of INI1 deletions in pGADNot has been described[14]. *Bam*HI-*Bgl*II fragment containing INI1 cDNA were isolated from clones 27B (aa1–245), 20.2 (aa141–385), S6 (aa183–294) and inserted into *Bam*H1 site of pCGN, to generate clones expressing various HA-fusions. Plasmid pGFP-INI1 expressing GFP fused to the N terminus of INI1, was generated by inserting a 1.3-kb *Eco*RI fragment of pSH2-INI1 into the *Eco*RI site of pEGFP-C2 (Clontech, Palo Alto, California). Plasmid pGFP-S6 and pGFP-E3 were generated by PCR using pGADNot-S6 and pGADNot-E3, respectively, as templates, and by using primers EC2 (5′-CCGCTCTAGATCAACCGAGGGC-3′) and EC7 (5′-CGAATTCCGGATGCGCGAGCCC-3′), and inserting the digested fragment at the *Xba*I and *Eco*RI sites of pEGFP-C2.

Transfection, viral-particle production and p24 assays. 293T cells at 30% confluency were transfected with a 2:1:1 ratio (20 μg) of transducing vector (pHR′CMV-GFP or pHR′CMV-LacZ), pMDG (expressing the VSVG envelope protein) and pCMVΔR8.2 (expressing Gag–pol) along with 20 μg of either pCGN-INI1, pCGN-S6 or pCGN-S6 mutants, using the calcium phosphate transfection method (Cat# S-001, Specialty Media, Phillipsburg, New Jersey) according to manufacturer's instructions. In MON cells, a total of 10 μg of three-plasmid–based vectors was used. Effect of INI1, S6 or E3 on the production of replication-competent molecular clone HIV-1_{R3B}, or three-plasmid–based vectors carrying H12Y mutation in integrase, or SG3-integrase virus carrying Vpr-RT-integrase, was also determined by co-transfecting the respective viral constructs with pCGN-INI1, pCGN-S6 or pCGN-E3 plasmids using calcium phosphate method as above. The p24 ELISA assays were carried out using the NEN kit (Cat # NEK-050A). Intracellular levels of p24 antigen in the transfected cells were determined by lysing the cells from a 10-cm plate in 0.2 ml RIPA buffer. The supernatant was filtered through a 0.2-μM acetate filter (Costar, Cambridge, Massachusetts; Cat# 8112) before use in p24 ELISA or in immunoblot analyses.

Generation of pools of Jurkat stable cell lines expressing HA-INI1 or HA-S6. Approximately 5×10^6 Jurkat cells were electroporated at 250 V and 1.07 μF, and plated out in 5 ml RPMI media (Gibco, Carlsbad, California) in 60-mm dishes. About 24 h post-electroporation, 1.25 mg/ml G-418 was added and selected for 18 d until all the untransfected cells were eliminated. Pools of resistant cells expressing either HA-INI1 or HA-S6 were amplified and were used as target cells for infection with HIV-1_{R3B}. The expression of the HA-INI1 and HA-S6 in these cells were verified by immunoblot analysis using antibodies against HA (data not shown).

Reverse two-hybrid analysis. A library of S6 mutants was generated by taking advantage of the low fidelity of *Taq* polymerase in the presence of manganese. PCR was carried out using pGADNot-S6 as the template, 5′-Gal and 3′-Gal oligonucleotides as primers, and *Taq* polymerase (NEB, Beverly, Massachusets) with an annealing temperature of 50 °C. The PCR fragments were then digested with *Bam*H1 and *Sal*I and cloned into pGADNot to generate a library of S6 mutants as fusions to GAL4-AC (activation domain). This library was screened against the bait plasmid, LexA-DB (DNA binding) fusion of HIV-1 integrase in yeast, and non-interacting mutants of S6 were isolated as those resulting in white colonies upon X-Galactosidase staining. Plasmids were rescued from the yeast colonies, re-tested and sequenced to determine the mutations. Four clones that contained single-point mutations were subcloned into a pCGN vector to express HA-fusion proteins in mammalian cells.

Co-immunoprecipitation studies. 293T cells were transiently transfected with 20 μg of either pCGN, pCGN-INI1 and pCGN-S6 along with SG3-integrase and Vpr-RT- integrase using the calcium phosphate method. 48 h post-transfection, the cells were collected and lysed in Buffer L (ref. 33) and pre-cleared with Protein A Sepharose, then incubated with polyclonal antibody against HA (Santa Cruz Biotech, Santa Cruz, California; Cat#SC-805). Protein-antibody complexes were subsequently incubated with Protein A Sepharose. Bound proteins were washed in Buffer L containing 125 mM NaCl, separated by SDS–PAGE and analyzed by immunoblot analysis using antibodies against integrase or INI1, and chemiluminescent detection.

Preparation of H12Y virions. A *Bcl*I-*Cla*I fragment of pCMVΔR8.2 was subcloned into pBluescipt to generate an intermediate vector. The H12Y substitution was introduced into the intermediate vector using the Quickchange method (Stratagene, La Jolla, California), and the entire region was sequenced to confirm the presence of H12Y mutation and the absence of any other mutations. The *Bcl*I-*Cla*I fragment containing H12Y mutation was subsequently cloned into the pCMVΔR8.2 vector, to generate pCMV-H12Y. This clone was transfected into 293T cells along with pHR′CMV-LacZ and pMDG for generating pseudotyped H12Y virions.

Sub-cellular localization of GFP-INI1/S6/E3. 293T cells were cultured and transfected on 12-mm circular cover-slips (0.13–0.17 mm in thickness) that had been autoclaved, using calcium phosphate method. 24 h after transfection, cells were fixed with 2% paraformaldehyde, washed and permeabilized in 1% Triton X-100, and then treated with 1 μg/ml RNase A before staining with propidium iodide (40 μg/ml). Cells were mounted on a glass slide and confocal images were captured on a BioRad MRC6000 machine (Richmond, California).

Acknowledgments

We thank V.R. Prasad and J. Brojatsch for critically reading the manuscript; R. Kim, R. Beltran, S-W. G. Cheng and P. Joshi for technical help; D. Helland and D. Trono for reagents; S. Goff for helpful discussions; and A. Rubinstein, Y. Mizrachi, M. Mantovani, G. Liu and N. Sokolov for assistance with the p24 ELISA assays. This work was funded by a NIH grant AI/GM 399951 to G.V.K. and a contract NOI-CO-56000 to D.O. M.S. and A.P. were funded by an institutional training grant T32-AI07501

RECEIVED 3 MAY; ACCEPTED 25 JUNE 2001

1. Pani, A. & Marongiu, M.E. Anti-HIV-1 integrase drugs: How far from the shelf? *Curr. Pharm. Des.* **6**, 569–584 (2000).
2. Pommier, Y. & Neamati, N. Inhibitors of human immunodeficiency virus integrase. *Adv. Virus Res.* **52**, 427–458 (1999).
3. Mathe, C. & Nair, V. Potential inhibitors of HIV integrase. *Nucleosides Nucleotides* **18**, 681–682 (1999).
4. Asante-Appiah, E. & Skalka, A.M. Molecular mechanisms of retroviral DNA integration. *Antiviral Res.* **36**, 139–156 (1997).
5. Brown, P.O. Integration of retroviral DNA. *Curr. Topics Microbiol. Immunol.* **157**, 19–48 (1990).
6. Desrosiers, R.C. Strategies used by human immunodeficiency virus that allow persistent viral replication. *Nature Med.* **5**, 723–725 (1999).
7. Brown, P. Integration. in *Retroviruses* (eds. Coffin, J.M., Hughes, S.H. & Varmus, H.E.) 161–203 (Cold Spring harbor Laboratory Press, Cold Spring Harbor, New York, 1997).
8. Engleman, A., Englund, G., Orenstein, J.M., Martin, M.A. & Craigie, R. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. *J. Virol.* **69**, 2729–2736 (1995).
9. Masuda, T., Planelles, V., Krogstad, P. & Chen, I.S.Y. Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 *att* site: Unusual phenotype of mutants in the zinc finger-like domain. *J. Virol.* **69**, 6687–6696 (1995).
10. Wu, X. *et al.* Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex. *J. Virol.* **73**, 2126–2135 (1999).
11. Leavitt, A.D., Robles, G., Alesandro, N. & Varmus, H.E. Human immunodeficiency virus type 1 integrase mutants retain *in vitro* integrase activity yet fail to integrate viral DNA efficiently during infection. *J. Virol.* **70**, 721–728 (1996).
12. Nakamura, T. *et al.* Lack of infectivity of HIV-1 integrase zinc finger-like domain mutant with morphologically normal maturation. *Biochem. Biophys. Res. Commun.* **239**, 715–722 (1997).
13. Kalpana, G.V., Marmon, S., Wang, W., Crabtree, G.R. & Goff, S.P. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. *Science* **266**, 2002–2006 (1994).
14. Morozov, A., Yung, E. & Kalpana, G. Structure-function analysis of integrase inter-

actor 1L1 reveals differential properties of two repeat motifs present in the highly conserved region. *Proc. Natl. Acad. Sci. USA* **95**, 1120–1125 (1998).
15. Versteege, I. *et al.* Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* **394**, 203–206 (1998).
16. Biegel, J.A. *et al.* Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors. *Cancer Res.* **59**, 74–79 (1999).
17. Wang, W. *et al.* Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *EMBO J.* **15**, 5370–82 (1996).
18. Kingston, R.E. & Narlikar, G.J. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev.* **13**, 2339–52 (1999).
19. Naldini, L. *et al. In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263–267 (1996).
20. Leung, D.W., Chen, E. & Goeddel, D.V. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Techique* **1**, 11–15 (1989).
21. Wu, X. *et al.* Functional RT and integrase incorporated into HIV-1 particles independently of the Gag/Pol precursor protein. *EMBO J.* **16**, 5113–22 (1997).
22. Adachi, A. *et al.* Generation and characterization of human immunodeficiency virus type 1 mutants. *Arch. Virol.* **117**, 45–58 (1991).
23. Ansari-Lari, M.A., Donehower, L.A. & Gibbs, R.A. Analysis of human immunodeficiency virus type 1 integrase mutants. *Virol.* **211**, 332–335 (1995).
24. Cannon, P.M., Wilson, M., Byles, E., Kingsman, S.M. & Kingsman, A.J. Human immunodeficiency virus type 1 integrase: Effect on viral replication of mutations at highly conserved residues. *J. Virol.* **68**, 4768–4775 (1994).
25. Gallay, P., Swingler, S., Song, J., Bushman, F. & Trono, D. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83**, 569–576 (1995).
26. Stevenson, M., Stanwick, T.L., Dempsey, M.P. & Lamonica, C.A. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* **9**, 1551–1560 (1990).
27. Wiskerchen, M. & Muesing, M.A. Human immunodeficiency virus type 1 integrase: Effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J. Virol.* **69**, 376–386 (1995).
28. Fletcher, T.M., 3rd *et al.* Complementation of integrase function in HIV-1 virions. *EMBO J.* **16**, 5123–38 (1997).
29. Muchardt, C., Sardet, C., Bourachot, B., Onufryk, C. & Yaniv, M. A human protein with homology to Saccharomyces cerevisiae SNF5 interacts with the potential helicase hbrm. *Nucleic Acids Res.* **23**, 1127–32 (1995).
30. Ott, D.E. *et al.* Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. *J. Virol.* **70**, 7734–7743 (1996).
31. Bukovsky, A. & Gottlinger, H. Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease. *J. Virol.* **70**, 6820–6825 (1996).
32. Swanstrom, R. & Wills, J.W. Synthesis, assembly, and processing of viral proteins. in *Retroviruses* (eds. Coffin, J.M., Hughes, S.H. & Varmus, H.E.) 263–334 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1997).
33. Cheng, S.-W. G. *et al.* c-MYC interacts with INII/hSNF5 and requires the SWI/SNF complex for transactivation function. *Nature Gen.* **22**, 102–105 (1999).